Description

MUTANT BACTERIUM BELONGING TO THE GENUS BACILLUS

Technical Field

[0001]

The present invention relates to a host microorganism and a recombinant microorganism, which can be employed to produce useful proteins and polypeptides, and to a method for producing such proteins and polypeptides.

Background Art

[0002]

Microorganisms are widely used for industrially producing a broad range of useful substances, including alcoholic beverages, certain types of foods such as *miso* (i.e., fermented soybean paste) and *shoyu* (i.e., soy sauce), amino acids, organic acids, nucleic-acid-related substances, antibiotics, sugars, lipids, and proteins. These substances also find diversified uses, including foods, pharmaceuticals, detergents, products for daily use such as cosmetics, and a variety of chemical raw materials.

[0003]

In industrial production of useful substances by use of microorganisms, improvement of productivity is one major topic of interest, and one approach therefor is breeding of microorganisms through mutagenesis or other genetic means.

Recently, in particular, with advancement of microbial genetics and biotechnology, more efficient production of useful substances through gene recombination techniques attracts attention. In a known method for breeding productive microorganisms by use of gene recombination techniques, a transcription factor which regulates gene expression, in particular an RNA polymerase sigma factor, is potentiated. For example, in *Pseudomonas fluorescens*, the number of copies of a *rpoD* gene encoding a primary sigma factor (housekeeping sigma factor), which participates in transcription of genes essential to the growth during the vegetative stage, is increased, to thereby increase the production amount of an antibiotic, such as pyoluteorin or 2,4-diacetylphloroglucinol (see, for example, Non-Patent Document 1), and in *Corynebacterium glutamicus*, housekeeping *sigA* gene is overexpressed, to thereby increase the amount of fermentive production of L-lysine (see, for example, Patent Document 1).

[0004]

In the above approaches, however, an increased expression of a housekeeping sigma factor is attained during the vegetative stage. Moreover, regarding microorganisms belonging to the genus *Bacillus*, such as *Bacillus subtilis*, no report has so far been published that states augmentation of a sigma factor leads to an increased production of useful substances.

Patent Document 1: WO 2003/054179

Non-Patent document 1: J. Bacteriol., 177, 5387, (1995)

Disclosure of the Invention

[0005]

The present invention provides a mutant bacterium produced from a bacterium belonging to the genus *Bacillus* (hereinafter referred to as "a mutant *Bacillus* bacterium"), the mutant *Bacillus* bacterium having, on the genome or a plasmid thereof, a DNA molecule which includes a *sigA* gene or a gene functionally equivalent thereto and a promoter sequence ligated to the upstream end of the gene, wherein the promoter sequence is recognized and transcribed specifically during the sporulation stage.

[0006]

The present invention also provides a recombinant microorganism created by transferring, to a mutant *Bacillus* bacterium, a gene encoding a heterologous protein or polypeptide, and a method for producing a protein or polypeptide through use of the recombinant microorganism.

[0007]

The present invention further provides a method of constructing a mutant *Bacillus* bacterium, characterized in that a microorganism belonging to the genus *Bacillus* is modified so as to have, as a genomic DNA or plasmid DNA, a DNA molecule which includes a *sigA* gene or a gene functionally equivalent thereto and a promoter sequence ligated to the upstream end of the gene, wherein the promoter

sequence is recognized and transcribed specifically during the sporulation stage of the microorganism.

Brief Description of the Drawings

[0008]

[Fig. 1] A chart showing sequential activation of sigma factors during the sporulation stage.

[Fig. 2] A conceptual diagram showing an exemplary process for constructing the sigA gene of the present invention.

Best Mode for Carrying Out the Invention

[0009]

The present invention is directed to a mutant bacterium which provides enhanced production of a protein or polypeptide; a recombinant microorganism created by transferring, to the mutant *Bacillus* bacterium, a gene encoding a heterologous protein or polypeptide; and a method for producing a protein or polypeptide through use of the recombinant microorganism.

[0010]

Microorganisms belonging to the genus Bacillus have a plurality of sigma factors, each being a subunit of RNA polymerase and participating in the recognition of a promoter sequence. It has been postulated that, when each of different sigma factors which recognize different promoters is bound to an RNA polymerase core complex composed of a

plurality of subunits which are not sigma factors, a different gene is transcribed, whereby for each of the thousands of genomic genes, expression is controlled as stipulated by specific conditions. For example, regarding *Bacillus subtilis* belonging to the genus Bacillus, 17 sigma factors have been identified. They include SigA (also called a housekeeping sigma factor), which is a primary sigma factor that participates in transcription of a gene which is essential for growth during the vegetative growth period; SigH, SigF, SigE, SigG, and SigK, which control sporulation; SigD, which controls flagellum biogenesis and cell wall lysis; SigL, which controls metabolism of certain amino acids or saccharides; SigB, which controls the ability of adjustment to environmental changes; and a sigma factor named ECF sigma (*Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp289, (2002)).

[0011]

Of the above sigma factors, those that control over the sporulation stage are known to be sequentially expressed and activated as the sporulation process advances as shown in Fig. 1. Specifically, when *Bacillus subtilis* falls into a shortage of nutrients, firstly, phosphorylation of Spo0A, a sporulation initiation control factor, occurs via a multi-step phosphate transport system——also called the phosphate relay system——provided for a plurality of proteins (Cell, 64, 545, (1991)). As the phosphorylated Spo0A (Spo0A-P)

level elevates, repressor AbrB, which represses expression of *sigH*, the structural gene of SigH, is less induced, and as a result, transcription of sigH is induced to become SigA-dependent (J. Bacteriol., 173, 521, (1991)). Once SigH has been activated, through asymmetrical membrane formation, the cytoplasm of Bacillus subtilis is divided into compartments of mother cell side and daughter cell side. Subsequently, on the daughter cell side, Spo0A-P and SigH cooperate to induce transcription of an operon (*spoIIAA-spoIIAB-sigF*) which contains *sigF*, a structural gene of SigF (Gene, 101, 113, (1991)), and on the mother cell side, Spo0A-P and SigA cooperate to induce transcription of an operon (*spoIIGA-sigE*) which contains *sigE*, a structural gene of a SigE precursor (J. Bacteriol., 169, 3329, (1987)). Activation of SigF is controlled by an anti-sigma factor SpoIIAB and an anti-anti-sigma factor SpoIIAA, and also by SpoIIE, which is a dephosphorylase for SpoIIAA (Genes Cells, 1, 881 (1996)). Activated SigF induces transcription of *spoIIR*, a structural gene of a signal transduction protein SpoIIR. SpoIIR secreted from the daughter cell side activates SpoIIGA, which is a SigE precursor activating protease localized in the asymmetrical membrane on the mother cell side, presumably leading to activation of SigE (Proc. Natl. Acad. Sci. U.S.A., 92, 2012, (1995)).

[0012]

Moreover, on the daughter cell side, SigF induces transcription of *sigG*, a structural gene of SigG, while on

the mother cell side, SigE induces transcription of sigK, a structural gene of SigK. Activation of SigG on the daughter cell side occurs after the SigE activation has taken place on the mother cell side. Afterwards, activation of SigK on the mother cell side occurs (Mol. Microbiol., 31, 1285, (1999)).

[0013]

During vegetative growth, through association with an RNA polymerase core complex, SigA is reported to predominantly direct transcription of a gene having a SigA-recognizable promoter, or an operon. During sporulation, when other sigma factors are activated through the above-mentioned mechanism, substitution takes place to replace the sigma factor that is associated with the RNA polymerase core complex, resulting in a relative decrease in the amount of SigA-associated RNA polymerase (J. Bacteriol., 179, 4969 (1999)). Thus, during and after the sporulation stage, the level of transcription from a SigA-recognized promoter is considered to decrease as compared with the level during the vegetative growth stage.

[0014]

Under such circumstances, the present inventors have found that, through ligating a promoter sequence recognized and expressed specifically during the sporulation stage to a gene encoding SigA which is a main sigma factor relating to transcription of genes essential in growth mainly during the vegetative stage, expression of the gene encoding SigA can be enhanced during the sporulation stage which follows the

vegetative stage and level of binding between the sigma factor and an RNA polymerase core complex can be enhanced, to thereby increase productivity of a heterologous protein or polypeptide after the sporulation stage can be enhanced.

[0015]

When the microorganism of the present invention is employed, the above heterologous protein or polypeptide can be produced efficiently.

[0016]

In the present invention, homology between amino acid sequences and that between nucleic acid sequences are both determined by use of the Lipman-Pearson method (Science, 227, 1435 (1985)). Specifically, calculation is performed by use of a homology analysis program (Search Homology) developed by genetic information processing software Genetyx-Win, (Software Development Co., Ltd.), with ktup (the unit size to be compared) being set to 2.

[0017]

The mutant *Bacillus* bacterium of the present invention is constructed such that DNA having a promoter sequence which is recognized and transcribed specifically during the sporulation stage and which is ligated to an upstream end of a *sigA* gene or a gene equivalent thereto is present on the genome or a plasmid thereof.

[0018]

No particular limitation is imposed on the origin of a parent microorganism employed for constructing such a mutant

Bacillus bacterium, so long as the parent microorganism is a bacterium belonging to the genus *Bacillus* exhibiting a unique feature of sporulation, and a wild type microorganism or a mutant microorganism may be employed. Preferred examples of bacteria belonging to the genus *Bacillus* employed in the present invention include *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus halodulans*, whose complete genomic information has already been obtained. In particular, *Bacillus subtilis* is preferred from the viewpoint that genetic engineering techniques and genomic engineering techniques for this microorganism have been established, and that the microorganism has ability to secrete the produced protein extracellularly.

[0019]

As used herein, a *sigA* gene of *Bacillus subtilis* refers to a gene encoding an amino acid sequence represented by SEQ ID NO: 1. A gene equivalent to the *sigA* gene refers to a gene encoding an amino acid sequence having a homology of 70% or more to the amino acid sequence represented by SEQ ID NO: 1, preferably 80% or more, more preferably 90% or more, still more preferably 95% or more, yet still more preferably 98% or more.

[0020]

In the present invention, a promoter sequence recognized and transcribed specifically during the sporulation stage is ligated to an upstream end of such a *sigA* gene or a gene equivalent thereto. The promoter

sequence, which is recognized and transcribed specifically during the sporulation stage, may be any of a naturally derived sequence, a modified naturally derived sequence, and a chemically synthesized sequence.

[0021]

Examples of the promoter sequences employed for creating a mutant *Bacillus subtilis* include a promoter sequence having a characteristic feature described in any one of (1) to (6).

(1) a promoter sequence whose transcription repression by AbrB is canceled as Spo0A-P level increases and which is recognized and transcribed by SigA

(2) a promoter sequence which is recognized and transcribed by SigH

(3) a promoter sequence which is recognized and transcribed by SigF

(4) a promoter sequence which is recognized and transcribed by SigE

(5) a promoter sequence which is recognized and transcribed by SigG

(6) a promoter sequence which is recognized and transcribed by SigK

[0022]

It is generally accepted that a sigma factor is bound to a sequence of several bases that is present in the vicinity of a 10-base upstream site or 35-base upstream site from the transcription start point. The sequences

corresponding to these sites are called the -10 region and the -35 region, respectively. Moreover, it has been known that, for each sigma factor, common characteristics are shared by the base sequence and the distance between the two regions. Thus, such a sequence is called a consensus sequence. Therefore, examples of the promoter sequences (1) to (6) include (1′) a sequence whose transcription repression by AbrB is canceled as Spo0A-P level increases and having a consensus sequence which is recognized by SigA, (2′) a sequence having a consensus sequence which is recognized by SigH, (3′) a sequence having a consensus sequence which is recognized by SigF, (4′) a sequence having a consensus sequence which is recognized by SigE, (5′) a sequence having a consensus sequence which is recognized by SigG, (6′) a sequence having a consensus sequence which is recognized by SigK.

The consensus sequences which have heretofore been reported for the sigma factors of *Bacillus subtilis* are shown in Table 1.

[0023]

[Table 1]

Sigma factor	Consensus sequence		
	-35 region	Distance between two regions	-10 region
SigA	TTGaca	14	tgnTAtaat
SigH	RnAGGwWW	11-12	RnnGAAT
SigF	GywTA	15	GgnrAnAnTw
SigE	Ata	16-18	cATAcanT
SigG	gnATr	15	cAtnnTA
SigK	AC	16-18	CATAnnnT

[0024]

(*Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp289, (2002))

In the above listed sequences, R denotes A or G, W denotes A or T, and N denotes any nucleotides, and when nucleotides are shown with upper case letters, the nucleotides are highly conserved, whereas when nucleotides are shown with lower case letters, the nucleotides are not well conserved.

[0025]

A sequence which is recognized and bound by AbrB, which have been reported, is represented by a nucleotide sequence of WaWWtttWCAAaaaaW (W denotes A or T, and when nucleotides are shown with upper case letters, the nucleotides are highly conserved, whereas when nucleotides are shown with lower case letters, the nucleotides are not well conserved (J. Bacteriol., 177, 6999, (1995)).

As described above, a promoter sequence recognized and transcribed specifically during the sporulation stage in the

present invention has a sequence of any one of the above (1) to (6) and (1') to (6').

[0026]

Examples of the promoter having a trait of (1) or (1') and originating from nature include promoters for a gene or an operon of *Bacillus subtilis* listed in Table 2; examples of the promoter having a trait of (2) or (2') and originating from nature include promoters for a gene or an operon of *Bacillus subtilis* listed in Table 3; examples of the promoter having a trait of (3) or (3') and originating from nature include promoters for a gene or an operon of *Bacillus subtilis* listed in Table 4; examples of the promoter having a trait of (4) or (4') and originating from nature include promoters for a gene or an operon of *Bacillus subtilis* listed in Table 5; examples of the promoter having a trait of (5) or (5') and originating from nature include promoters for a gene or an operon of *Bacillus subtilis* listed in Table 6; and examples of the promoter having a trait of (6) or (6') and originating from nature include promoters for a gene or an operon of *Bacillus subtilis* listed in Table 7.

[0027]

The names, numbers, and functions of respective genes in the Tables contained herein conform with the *Bacillus subtilis* genome data reported in Nature, 390, 249 to 256 (1997) and made public by JAFAN (Japan Functional Analysis Network for *Bacillus subtilis*; BSORF DB) on the Internet (http://*bacillus*.genome.ad.jp/ renewed June 17, 2003).

[0028]

[Table 2]

Gene name	Gene No.
sigH	BG10159
spo0E	BG10769
aprE	BG10190
sinI	BG10753
dppA	BG10842
abrB	BG10100
ftsA	BG10231
pbpE	BG10390
kinB	BG10745

[0029]

(*Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics, Edited by A. L. Sonenshein, American Society for Microbiology, pp757, (1993), J. Bacteriol., 177, 6999, (1995))

In the above Table, when an operon is referred to, the name of the first gene of the transcription unit is given.

[0030]

[Table 3]

Gene name	Gene No.
sigA	BG10314
spo0M	BG12229
spoVG	BG10112
citG	BG10384
spo0F	BG10411
spoVS	BG11245
ureA	BG11981
yvyD	BG10740
spo0A	BG10765
ftsA	BG10231
kinA	BG10204
spoIIAA	BG10296
minC	BG10329
phrC	BG11959
ytxG	BG10974

[0031]

(*Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp 289, (2002))

In the above Table, when an operon is referred to, the name of the first gene of the transcription unit is given.

[0032]

[Table 4]

Gene name	Gene No.
dacF	BG10295
bofC	BG11917
gerAA	BG10385
gpr	BG10438
katX	BG11945
sspN	BG14179
spoIIQ	BG11978
spoIIR	BG10937
spoIIIG	BG10236
spoIVB	BG10311
ywhE	BG12459
yhcN	BG11592
lonB	BG11077

[0033]

(*Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp 289, (2002))

In the above Table, when an operon is referred to, the name of the first gene of the transcription unit is given.

[0034]

[Table 5]

Gene name	Gene No.
spoIIP	BG10439
spoIID	BG10766
spoIIM	BG10768
bofA	BG10087
spoIIIAA	BG10540
spoIIID	BG10408
spoIVFA	BG10331
cotE	BG10494
cotJA	BG11799
dacB	BG10527
spoIVA	BG10275
spoIVCB	BG10459
spoVB	BG10778
spoVD	BG10222
spoVE	BG10226
spoVK	BG11039
spoVM	BG10776
spoVR	BG10182
spoVID	BG10346
glgB	BG10907
mmgA	BG11319
phoB	BG10697
yknT	BG12251
yteV	BG12339
safA	BG13781
yaaH	BG10080
cwlD	BG11514
cwlJ	BG11172
yjmC	BG13206
yfhS	BG12892
yoaW	BG13493

[0035]

(*Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp 289, (2002))

In the above Table, when an operon is referred to, the name of the first gene of the transcription unit is given.

[0036]

[Table 6]

Gene name	Gene No.
gerAA	BG10385
gerBA	BG10640
gerD	BG10644
csgA	BG11504
bofC	BG11917
dacF	BG10295
gpr	BG10438
spoVAA	BG10892
spoIIIG	BG10236
spoVT	BG10119
sspA	BG10786
sspB	BG10787
sspC	BG10882
sspD	BG10788
sspE	BG10789
sspF	BG10108
sspJ	BG14174
sleB	BG11439
splA	BG10202
sspN	BG14179
spoIVB	BG10311
sspH	BG12917
sspL	BG14176
ybaK	BG11503
yhcN	BG11592
ywhE	BG12459
ycxE	BG11066
sspI	BG12318
sspK	BG14175
sspM	BG14177
sspO	BG11920
cwlD	BG11514

[0037]

(*Bacillus subtilis* and Its Closest Relatives: From Genes to Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp 289, (2002))

In the above Table, when an operon is referred to, the name of the first gene of the transcription unit is given.

[0038]

[Table 7]

Gene name	Gene No.
cgeA	BG11193
cgeC	BG11195
cwlC	BG10825
cotA	BG10490
cotB	BG10491
cotC	BG10492
cotD	BG10493
cotE	BG10494
cotF	BG10012
cotH	BG11791
cotM	BG11822
cotT	BG10495
cotG	BG11017
cotSA	BG11381
cotV	BG10496
cotX	BG10500
cotY	BG10498
yobW	BG12269
yqeE	BG11633
spoIVCB	BG10459
spoVK	BG11039
spoVFA	BG10781
gerE	BG10355
sspG	BG14173
yfhP	BG12890
yabG	BG10106

[0039]

(*Bacillus subtilis* and Its Closest Relatives: From Genes to

Cells, Edited by A. L. Sonenshein, American Society for Microbiology, pp 289, (2002))

In the above Table, when an operon is referred to, the name of the first gene of the transcription unit is given.

[0040]

As described above, preferred examples of the promoter sequence recognized and transcribed during the sporulation stage employed in the present invention include promoters having a sequence of any one of (1) to (6) and (1') to (6'). In *Bacillus subtilis*, it is reported that SigE exhibits higher affinity with RNA polymerase as compared with SigA (J. Bacteriol., 179, 4969, (1999)). Therefore, more preferably, a promoter whose transcription is activated prior to activation of SigE is employed. Examples of more preferred promoter sequences include a promoter sequence whose transcription repression by AbrB is canceled as Spo0A-P level increases and which is recognized and transcribed by SigA (namely, (1) or (1')) and a promoter sequence which is recognized and transcribed by SigH (namely, (2) or (2')).

[0041]

Examples of the promoter having a sequence of (1) or (1') and originating from nature include promoter sequences for expressing a gene or an operon of *Bacillus subtilis* listed in Table 1. Of these, preferred promoters include a promoter sequence for a *sigH* of *Bacillus subtilis*. The promoter sequence for the *sigH* of *Bacillus subtilis* contains, in the nucleotide sequence represented by SEQ ID NO: 2, a

nucleotide sequence ranging from base Nos. 987 to 1,027, preferably a nucleotide sequence ranging from base Nos. 987 to 1047, more preferably a nucleotide sequence ranging from base Nos. 1 to 1,047; has a base length of 5,000 bp or less, preferably 2,000 bp or less, more preferably 1,047 bp or less; and has promoter functions equivalent to those of a promoter for expressing the *sigH* of *Bacillus subtilis*.

[0042]

Examples of the promoter having a sequence of (2) or (2') and originating from nature include promoter sequences for expressing a gene or an operon of *Bacillus subtilis* listed in Table 2. Of these, preferred promoters include a promoter sequence for expressing a *spoIIAA-spoIIAB-sigF* operon (*spoIIA* operon) of *Bacillus subtilis*. The promoter sequence for expressing the *spoIIA* operon of *Bacillus subtilis* contains, in the nucleotide sequence represented by SEQ ID NO: 3, a nucleotide sequence ranging from base Nos. 1,081 to 1,110, preferably a nucleotide sequence ranging from base Nos. 1,081 to 1,118, more preferably a nucleotide sequence ranging from base Nos. 1 to 1,143; has a base length of 5,000 bp or less, preferably 2,000 bp or less, more preferably 1,143 bp or less; and has promoter functions equivalent to those of a promoter for expressing the *sigH* of *Bacillus subtilis*.

[0043]

The promoter sequence recognized and expressed specifically during the sporulation stage employed in the

present invention includes a sequence virtually equivalent to the promoter sequence for expressing a gene or an operon of *Bacillus subtilis* listed in Tables 1 to 6. For example, a sequence corresponding to the promoter sequence for expressing the *sigH* of *Bacillus subtilis* includes a DNA fragment containing, in the nucleotide sequence represented by SEQ ID NO: 2, a nucleotide sequence ranging from base Nos. 987 to 1,027, preferably a nucleotide sequence ranging from base Nos. 1,081 to 1,118, more preferably a nucleotide sequence ranging from base Nos. 1 to 1,143, these sequences having one or more substituted, deleted, or inserted nucleotides; having a base length of 5,000 bp or less, preferably 2,000 bp or less, more preferably 1,047 bp or less; and having promoter functions equivalent to those of a promoter for expressing the *sigH* of *Bacillus subtilis*.

[0044]

Also, a sequence corresponding to the promoter sequence for expressing the *spoIIA* operon includes a DNA fragment containing, in the nucleotide sequence represented by SEQ ID NO: 3, a nucleotide sequence ranging from base Nos. 1,081 to 1,110, preferably a nucleotide sequence ranging from base Nos. 1,081 to 1,118, more preferably a nucleotide sequence ranging from base Nos. 1 to 1,143, these sequences having one or more substituted, deleted, or inserted nucleotides; having a base length of 5,000 bp or less, preferably 2,000 bp or less, more preferably 1,118 bp or less; and having promoter functions equivalent to those of a promoter for expressing the above-

mentioned operon.

[0045]

The promoter sequence employed in the present invention which is recognized by a sigma factor relating specifically to transcription during the sporulation stage includes promoter sequences for expressing ortholog genes of a gene of *Bacillus subtilis* or each of some genes constituting an operon of *Bacillus subtilis*, listed in Tables 2 and 3. The ortholog genes are preferably derived from bacteria belonging to the genus *Bacillus*. The ortholog genes can be located by use of a Create/view Orthologous gene table program of Microbial Genome Database (MBGD, http://mbgd.genome.ad.jp/) published on the internet. Examples of the ortholog genes of the *sigH* gene of *Bacillus subtilis* include a *sigH* (BH0115) gene of *Bacillus halodulans* and a BC0114 gene of *Bacillus cereus*. Examples of the ortholog genes of each of some genes constituting the *spoIIA* operon of *Bacillus subtilis* include a *sigF* (BH1538) gene, a *spoIIAB* (BH1537) gene, and a *spoIIAA* (BH1536) gene, these three genes being of *Bacillus halodulans;* and a BC4072 gene, a BC4073 gene, and a BC4074 gene, these three genes being of *Bacillus cereus*.

[0046]

As a promoter sequence which is recognized by a sigma factor participating specifically in transcription at the sporulation stage, the above promoter sequences may be employed singly or in combination of two or more species.

[0047]

DNA having a promoter sequence which is recognized and transcribed specifically during the sporulation stage and which is ligated to an upstream end of the *sigA* gene of *Bacillus subtilis* or a gene equivalent thereto can be constructed on the genome thereof through inserting a DNA fragment containing the promoter sequence recognized and transcribed specifically during the sporulation stage to an upstream site or downstream site of a SigA-recognized promoter sequence which is present at an upstream end of the *sigA* gene originally present on the genome of *Bacillus subtilis*. The DNA fragment containing the promoter sequence recognized and transcribed specifically during the sporulation stage may be inserted into any site which is located upstream of the *sigA* gene or a gene equivalent thereto. Preferably, the site is located in a region of 2,000 bp or less flanking an upstream side of a *sigA* structural gene, more preferably a region of 1,000 bp or less, still preferably a region of 500 bp or less, yet preferably a region of 1 to 198 bp. When the DNA fragment, which contains the promoter sequence recognized and transcribed specifically during the sporulation stage, has no proper sequence to be bound by ribosomes, the DNA fragment is preferably inserted into a region of 15 bp or more upstream of the *sigA* structural gene.

[0048]

Alternatively, the aforementioned DNA, which has a promoter sequence recognized and transcribed specifically

during the sporulation stage and which is ligated to an upstream end of the *sigA* gene or a gene equivalent thereto, can be constructed through PCR or other methods. Preferably, a sequence between a site ligated by the DNA fragment and the *sigA* structural gene; i.e., a sequence located upstream of the *sigA* gene which is originally present in the genome of *Bacillus subtilis*, has 0 to 2000 base pairs, more preferably 0 to 1000 base pairs, still preferably 0 to 500 base pairs, yet preferably 0 to 198 base pairs. When the DNA fragment, which has the promoter sequence recognized and transcribed specifically during the sporulation stage, contains no proper sequence to be bound by ribosomes, the above sequence between a site ligated by the DNA fragment and the *sigA* structural gene; i.e., a sequence located upstream of the *sigA* gene which is originally present in the genome of *Bacillus subtilis*, has preferably 15 base pairs or more. In order to construct the mutant *Bacillus* bacterium of the present invention, DNA which is constructed through the above method may be introduced into a parent bacterium belonging to the genus *Bacillus*.

[0049]

For example, a DNA fragment having a promoter sequence recognized and transcribed specifically during the sporulation stage may be ligated to a DNA fragment having a *sigA* gene and other genes through PCR; the thus-produced DNA fragment may be introduced into a plasmid vector capable of replicating in a parent bacterium belonging to the genus

Bacillus through cloning; and the plasmid vector may be introduced into the parent bacterium belonging to the genus *Bacillus*. When *Bacillus subtilis* is employed as the parent bacterium belonging to the genus *Bacillus* employed for constructing the mutant *Bacillus* bacterium of the present invention, a variety of plasmid vectors, which had been reported, capable of replicating in a *Bacillus subtilis* cell may be employed. Examples of the plasmid vectors include pUB110 (Plasmid, 15, 93, (1986)), pC194 (J. Bacteriol., 150, 815, (1982)), and pTX14-3 (Plasmid, 30, 119, (1993)).

[0050]

Alternatively, homologous recombination or similar techniques may be used to introduce, to the genome, a DNA fragment produced by ligating a DNA fragment containing a promoter sequence specifically recognized and transcribed during the sporulation stage to an upstream site of a DNA fragment containing, for example, a sigA gene. Several methods have already been reported for introducing a DNA fragment into the genome through homologous recombination (for example, Mol. Gen. Genet., 223, 268 (1990)). The mutant *Bacillus* bacterium may be produced through these methods.

[0051]

Next will be described in more detail a method for ligating a DNA fragment containing a promoter sequence recognized and transcribed during the sporulation stage to a DNA fragment containing a *sigA* gene through the SOE (splicing by overlap extension)-PCR method (Gene, 77, 51, 1989), to

thereby prepare a DNA fragment; and introducing the thus-prepared DNA fragment on the genome through homologous recombination. However, in the present invention, the method for introducing the DNA fragment is not limited only to the below-described method.

[0052]

In the present invention, in the first PCR, the following three fragments are prepared: a DNA fragment containing a promoter sequence recognized and transcribed specifically during the sporulation stage, a structural gene fragment of a housekeeping sigma factor, and a drug resistant marker gene. The primers to be used in this step may, for example, be those specifically designed so that an upstream 10 to 30 base pair sequence of the structural gene fragment of a housekeeping sigma factor is added to the downstream end of the DNA fragment containing the promoter sequence, and a downstream 10 to 30 base pair sequence of the structural gene fragment of the housekeeping sigma factor is added to the upstream end of the drug resistance marker gene (Fig. 2).

[0053]

Next, using three PCR fragments prepared in the first PCR as templates, the second PCR is performed by use of an upstream primer of the fragment containing the promoter sequence and a downstream primer of the drug resistance marker gene fragment. As a result, one end of *sigA* gene fragment anneals with the downstream end of the fragment containing the promoter sequence through the overlapping

sequences, and the other end of sigA gene fragment anneals with the upstream end of the drug resistance marker gene fragment through the overlapping sequences. Through PCR amplification, there can be obtained a DNA fragment in which the promoter sequence, which is recognized by a sigma factor relating specifically to transcription during the sporulation stage, is ligated to an upstream end of the *sigA* gene and the drug resistance marker gene is ligated downstream thereto (Fig. 2).

[0054]

When a *sigH* gene of *Bacillus subtilis* or a promoter for expressing the *spoIIA* operon is employed as a promoter recognized and transcribed specifically during the sporulation stage and when a chloramphenicol-resistant gene is employed as a drug resistance marker gene, a target DNA fragment can be obtained through SOE-PCR under typical conditions described in literature (see, for example, PCR Protocols. Current Methods and Applications, Edited by B. A. White, Humana Press, pp. 251 (1993), Gene, 77, 61, 1989), by use of a primer set such as that shown in Table 8 and a conventional enzyme kit for PCR (e.g., Pyrobest DNA Polymerase (Takara Shuzo)).

[0055]

The thus-obtained DNA fragment may be introduced into the genome of, for example, *Bacillus subtilis* as follows. The DNA fragment is introduced into a plasmid vector incapable of replicating in a *Bacillus subtilis* cell (e.g.;

pMW219 (NIPPON GENE)) through cloning; the plasmid vector is introduced into cells through the competent method or any suitable method, to thereby cause homologous recombination between a gene region encoding a housekeeping sigma factor on the plasmid and a *sigA* gene region on the genome; and, by use of a drug resistance marker as an indicator, there can be selected cells having on the genome the DNA fragment containing a *sigA* gene ligated to a promoter recognized and transcribed specifically during the sporulation stage and a plasmid vector (Fig. 2). Specifically, when a DNA fragment which had been prepared by use of a primer set shown in Table 3 is introduced into pMW219 through cloning, and the thus-obtained plasmid is introduced into a *Bacillus subtilis* cell, colonies grown in an agar medium supplemented with chloramphenicol are separated; and through PCR employing genomic DNA as a template or any suitable method, introduction, onto the genome, of the DNA fragment having the *sigA* gene ligated to a promoter region of the *sigH* gene or the spoIIA operon can be confirmed

[0056]

In the above procedure, *Bacillus subtilis* is employed as a parent bacterium belonging to the genus *Bacillus*. However, alternatively the mutant *Bacillus* bacterium of the present invention may be obtained in a similar manner from other bacteria belonging to the genus *Bacillus*.

[0057]

Using a mutant *Bacillus* bacterium produced in the

aforementioned manner, expression of SigA which transcribes genes encoding heterologous proteins or polypeptides, and a variety of genes relating to production of proteins can be enhanced, leading to improvement in production of the heterologous proteins or polypeptides.

Specifically, genes encoding target proteins or polypeptides are ligated downstream of a promoter recognized by SigA, and the product is introduced into the mutant *Bacillus* bacterium of the present invention, whereby the target proteins or polypeptides is produced not only during the vegetative stage but also during the sporulation stage, resulting in enhanced production of the target proteins or polypeptides, as compared with a parent bacterium belonging to the genus *Bacillus*.

[0058]

No particular limitation is imposed on the gene encoding the target protein or polypeptide. Examples of the protein and polypeptide include physiologically-active peptides and enzymes for industrial purposes such as detergents, foods, fibers, feeds, chemicals, medicine, and diagnostic agents. Industrial enzymes may be functionally grouped into oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases/synthetases. Preferably, hydrolases such as cellulase, α-amylase, and protease may be used. Specific examples include cellulase belonging to family 5 in the classification of enzymes which hydrolyze polysaccharides (Biochem. J., 280, 309, (1991)); in

particular, cellulase derived from a microorganism, more particularly cellulase derived from the genus *Bacillus*. Examples include alkaline cellulase having an amino acid sequence represented by SEQ ID NO: 4 which is derived from *Bacillus* sp. KSM-S237 (FERM BP-7875), alkaline cellulase having an amino acid sequence represented by SEQ ID NO: 6 which is derived from *Bacillus* sp. KSM-64 (FERM BP-2886), and cellulase which has another amino acid sequence having a homology of 70% to said amino acid sequence, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, or still further preferably 98% or more.

[0059]

Specific examples of α-amylase include α-amylase derived from a microorganism, preferably liquefied amylase derived from the genus *Bacillus*. Examples include alkaline amylase having an amino acid sequence represented by SEQ ID NO: 19 which is derived from *Bacillus* sp. KSM-K38 (FERM BP-6946), and amylase which has another amino-acid sequence having a homology of 70% or more to said amino-acid sequence, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, or even more preferably 98% or more. The homology of the amino-acid sequence is calculated by the Lipman-Pearson method (Science, 227, 1435 (1985)). Specific examples of protease include serine protease and metalloprotease which are derived from microorganisms, particularly those belonging to the genus *Bacillus*. Examples include alkaline protease having an amino acid sequence

represented by SEQ ID NO: 21 which is derived from *Bacillus clausii* KSM-K16 (FERM BP-3376), and protease which has another amino-acid sequence having a homology of 70% or more to said amino-acid sequence, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, or even more preferably 98% or more.

[0060]

As described above, the promoter sequence recognized by a housekeeping sigma factor (e.g., SigA of *Bacillus subtilis*) must be ligated to an upstream end of the gene of the target protein or polypeptide. In addition, preferably, regulatory regions related to translation or secretion; i.e., a site bound by ribosomes, a translation initiation region including the initiation codon, and a secretion signal peptide region, are properly ligated to the gene of the target protein or polypeptide. In one preferred example, a transcription initiation regulatory region containing a promoter transcribed by a housekeeping sigma factor, a translation initiation region, and a secretion signal peptide region of a cellulase gene derived from a microorganism belonging to the genus *Bacillus* disclosed in, for example, JP-A-2000-210081 or JP-A-1992-190793; i.e., a cellulase gene derived from KSM-S237 (FERM BP-7875) or KSM-64 (FERM BP-2886), are properly ligated to a structural gene of the target protein or polypeptide. More specifically, preferred DNA fragments to be ligated include a nucleotide sequence ranging from base Nos. 1 to 659 in SEQ ID NO: 5; a nucleotide sequence ranging

from base Nos. 1 to 696 of a cellulase gene represented by SEQ ID NO: 7; a DNA fragment having a nucleotide sequence having a homology of 70% or more to any one of said nucleotide sequences, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, even more preferably 98% or more; or a DNA fragment having a nucleotide sequence lacking a portion of any one of said nucleotide sequences. Preferably, one of these DNA fragments is properly ligated to a structural gene of the target protein or polypeptide.

[0061]

Productivity of the target protein or polypeptide can be enhanced by transferring a recombinant plasmid having a DNA fragment including a gene encoding the target protein or polypeptide ligated to a proper plasmid vector into the mutant *Bacillus* bacterium of the present invention through a conventional transformation technique. Alternatively, the DNA fragment is ligated to a proper region which is homologous with the genome of the mutant *Bacillus* bacterium of the present invention, to thereby a DNA fragment. The DNA fragment is inserted directly into the genome of the mutant *Bacillus* bacterium of the present invention, whereby productivity of the target protein or polypeptide may be enhanced.

[0062]

The target protein or polypeptide obtained by use of the mutant *Bacillus* bacterium of the present invention as a

host cell may be produced in such a manner that corresponding cells are inoculated onto a culture medium containing assimilable carbon sources and nitrogen sources, and other essential components; the cells are cultured through a conventional microorganism culturing method; and subsequently, protein or polypeptide is collected and purified.

[0063]

In the aforementioned manner, a bacterium belonging to the genus *Bacillus*, which exhibits enhanced efficiency in transcription of the *sigA* gene during the sporulation stage, can be constructed. When the mutant *Bacillus* bacterium is employed as a host cell for production through a recombinant technique, useful proteins and polypeptides may be produced efficiently.

[0064]

Next, a method for constructing the mutant bacterium belonging to genus *Bacillus* of the present invention and a method for producing cellulase by use of the same mutant bacterium as a host cell will be described in detail by way of Examples.

Examples

[0065]

Example 1 Construction of plasmid employed in introducing, into the genome of *Bacillus subtilis*, a *sigA* gene containing promoter transcribed specifically during the sporulation stage

Construction of a plasmid was performed. The plasmid was employed in introducing a DNA fragment having a promoter for expressing a *sigH* gene or a promoter for expressing a *spoIIA* operon ligated to an upstream end of a *sigA* structural gene into the genome of *Bacillus subtilis* through single crossing over homologous recombination in accordance with the procedure as shown in Fig. 2. Specifically, a genome DNA sample, serving as a template, extracted from *Bacillus subtilis* 168 and a primer set of sigAf and sigAr shown in Table 8 were employed, to thereby prepare a 1.2 kb fragment (A) having a *sigA* gene through PCR. In the same manner above, a primer set of sigHUf and sigHUr-sigA shown in Table 8 was employed, to thereby prepare a 1.0 kb fragment (B) containing a *sigH* gene promoter flanking the upstream side of the *sigH* gene on the genome. In the same manner above, a primer set of sigFUf and sigFUr-sigA shown in Table 8 was employed, to thereby prepare a 1.1 kb fragment (C) containing a *spoIIA* operon promoter flanking the upstream region of the *spoIIA* operon on the genome and controlling transcription of a sigF gene. A plasmid pC194 (J. Bacteriol. 150 (2), 815 (1982)) serving as a template and a primer set of CmFW and Cmr-sigA shown in Table 8 were used to prepare a 0.9 kb fragment (D) containing a chloramphenicol-resistant gene. Subsequently, SOE-PCR was performed by use of a primer set of sigHUf and CmFW shown in Table 8, and the thus-prepared three fragments (A), (B), and (D) in combination as templates, to thereby produce a 3.1 kb DNA fragment (E) in which the three

fragments (B), (A), and (D) were ligated in this sequence; i.e., the promoter for expressing the *sigH* gene was ligated to an upstream end of the *sigA* structural gene, and the chloramphenicol-resistant gene was ligated, in a reverse direction, downstream of the *sigA* structural gene. In the same manner, SOE-PCR was performed by use of a primer set of sigFUf and CmFW shown in Table 8, and by use of the three fragments (A), (C), and (D) in combination as templates, to thereby prepare a 3.2 kb DNA fragment (F) in which the three fragments (C), (A), and (D) were ligated in this sequence; i.e., the *spoIIA* operon promoter was ligated to an upstream end of the *sigA* structural gene, and the chloramphenicol-resistant gene was ligated, in a reverse direction, downstream of the *sigA* structural gene. The thus-prepared 3.1 kb DNA fragment (E) and 3.2 kb DNA fragment (F) were individually inserted into the *Sma*I restriction enzyme cleavage site of pMW219, which is a plasmid vector for E. coli and unable to replicate in the *Bacillus subtilis* cell, to thereby construct plasmids pMWPHsigA and pMWPFsigA employed in introducing, into the genome of *Bacillus subtilis*, the *sigA* gene containing the promoter transcribed specifically during the sporulation stage.

[0066]

The above procedure was repeated, except that primers sigAmf and sigFUr-sigAm shown in Table 8 was employed instead of the primers sigAf and sigFUr-sigA, to thereby construct pMWPFsigAm in which an initiation codon (ATG) of the *sigA*

gene in pMWPFsigA was substituted with a codon (ATA) which was not recognized as an initiation codon.

[0067]

Example 2 Introduction of the *sigA* gene containing promoter transcribed specifically during the sporulation stage into the genome of *Bacillus subtilis* 168

Bacillus subtilis 168 was transformed through the competent method with the plasmid pMWPHsigA, pMWPFsigA, or pMWPFsigAm. The plasmids pMWPHsigA and pMWPFsigA were employed for introducing, into the genome of *Bacillus subtilis*, the *sigA* gene containing the promoter transcribed specifically during the sporulation stage. The plasmid pMWPFsigAm was employed for introducing, into the genome of *Bacillus subtilis*, a modified *sigA* gene (*sigAm*) containing the same promoter, in which an initiation codon (ATG) of the *sigA* gene had been substituted by a codon (ATA). Colonies grown in an LB agar medium containing chloramphenicol were collected as transformants. Subsequently, the genome of the each transformant produced by use of pMWPHsigA or pMWPFsigA, serving as a template, was extracted, and PCR was performed thereon, to thereby confirm that the *sigA* gene containing the promoter transcribed specifically during the sporulation stage was inserted, together with the 1.2 kb fragment (D), into the genome through homologous recombination between the *sigA* gene of the genome and the *sigA* gene of pMWPHsigA or pMWPFsigA. In a similar manner, the genome of the each transformant produced by use of pMWPFsigAm was employed as

serving as a template, to thereby confirm that the *sigAm* was inserted, together with the 1.2 kb fragment (D), into the genome through homologous recombination between the *sigA* gene of the genome and the *sigAm* of pMWPFsigAm. The three transformants produced by use of pMWPHsigA, pMWPFsigA, and pMWPFsigAm, respectively, were denominated 168PHsigA, 168PFsigA, and 168PFsigAm.

[0068]

Example 3 Evaluation of *Bacillus subtilis* mutant strain in terms of alkaline cellulase production (secretion) performance

To each of the three types of *Bacillus subtilis* mutant strains obtained in Example 2 (168PHsigA, 168PFsigA, and 168PFsigAm) and to *Bacillus subtilis* 168 serving as a control, a recombinant plasmid pHY-S237 was introduced through the protoplast transformation method. The recombinant plasmid pHY-S237 was prepared by inserting a DNA fragment (3.1 kb) encoding an alkaline cellulase (JP-A-2000-210081) derived from *Bacillus* sp. KSM-S237 (FERM BP-7875) into the restriction enzyme *Bam*HI cleavage site of a shuttle vector pHY300PLK (yakult). The cells were shake-cultured in LB medium (10 mL) overnight at 37°C. The culture broth (0.05 mL) was inoculated to a 2 × L-maltose medium (50 mL) (2% tryptone, 1% yeast extract, 1% NaCl, 7.5% maltose, 7.5 ppm manganese sulfate 4-5 hydrate, and 15 ppm tetracycline), followed by shake-culturing at 30°C for three days. After completion of culturing, cells were removed through

centrifugation, and alkaline cellulase activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the alkaline cellulase secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline cellulase. As is clear from Table 9, more effective production, or secretion, of alkaline cellulase has been confirmed in the case where 168PHsigA or 168PFsigA was employed as a host cell, as compared with the control strain 168 (wild type). Meanwhile, equivalent secretion of alkaline cellulase has been confirmed in the case where 168PFsigAm was employed as a host cell, as compared with the control strain 168 (wild type). Thus, since the *sigA* gene, which was newly introduced into the genome, containing the promoter for expressing the *sigH* gene or the promoter for expressing the *spoIIA* operon was expressed to thereby produce SigA, more effective production may be attained in the case where 168PHsigA or 168PFsigA was employed.

[0069]

[Table 8]

Primer	Nucleotide sequence	SEQ ID NO.
SigAf	ATGGCTGATAAACAAACCCA	8
SigAr	CACCACAATGTTCATTTGCA	9
sigHUf	ACAGCCTTTCTTCCTCATTCT	10
sigHUr-sigA	CGTGGGTTTGTTTATCAGCCATTCCGATCCCCCCGGCGCACG	11
sigFUf	GCTGATAGAACGTGACACGGG	12
sigFUr-sigA	CGTGGGTTTGTTTATCAGCCATGCTCATTCCTCCTTGATATG	13
CmFW	CAACTAAAGCACCCATTAG	14
Cmr-sigA	CATTTGCAAATGAACATTGTGGTGCTTCTTCAACTAACGGGGCA	15
sigAmf	ATAGCTGATAAACAAACCCA	16
sigFUr -sigAm	CGTGGGTTTGTTTATCAGCTATGCTCATTCCTCCTTGATATG	17

[0070]

[Table 9]

Host	Amount of produced (secreted) alkaline cellulase (relative value)
168 (wild type strain)	100
168PHsigA	135
168PFsigA	146
168PFsigAm	108

[0071]

Example 4 Evaluation of *Bacillus subtilis* mutant strain in terms of alkaline protease production (secretion) performance

168PHsigA and 168PFsigA, exhibiting enhanced alkaline cellulase productivity confirmed in Example 3, were evaluated in terms of production performance of other proteins and polypeptides. Specifically, among others, alkaline protease production performance of the above two strains was investigated by use of the genus *Bacillus* in the following procedure.

A genome DNA sample, serving as a template, extracted from *Bacillus clausii* KSM-K16 (FERM BP-3376) and a primer set of S237pKAPpp-F and KAPter-R (*Bgl*II) shown in Table 10 were employed, to thereby amplify through PCR a 1.3 kb DNA fragment (G) encoding alkaline protease (Appl. Microbiol. Biotechnol., 43, 473, (1995)) having an amino acid sequence represented by SEQ ID NO: 21. Separately, a genome DNA sample, serving as a template, extracted from *Bacillus* sp. KSM-S237 (FERM BP-7875) and a primer set of S237ppp-F2

(*Bam*HI) and S237pKAPpp-R shown in Table 10 were employed, to thereby amplify through PCR a 0.6 kb DNA fragment (H) containing a promoter region of an alkaline cellulase gene (JP-A-2000-210081). Subsequently, SOE-PCR was performed by use of a primer set of S237ppp-F2 (*Bam*HI) and KAPter-R (*Bgl*II) shown in Table 10 and the thus-obtained two fragments (G) and (H) in combination as templates, to thereby produce a 1.8 kb DNA fragment (I) in which an alkaline protease gene was ligated to downstream of the promoter region of an alkaline cellulase gene. The thus-produced 1.8 kb DNA fragment (I) was inserted into the *Bam*HI-*Bgl*II restriction enzyme cleavage site of a shuttle vector pHY300PLK (yakult), to thereby construct a plasmid pHYKAP (S237p) for evaluating alkaline protease productivity.

The thus-constructed plasmid pHYKAP (S237p) was introduced to each of the 168PHsigA and 168PFsigA, and to *Bacillus subtilis* 168 serving as a control through the protoplast transformation method. The cells were shake-cultured for three days, and other conditions were the same as employed in Example 3. After completion of culturing, cells were removed through centrifugation, and alkaline protease activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the alkaline protease secreted from the cells during culturing; i.e., the amount of the extracellularly produced alkaline protease. As is clear from Table 11, more effective production, or secretion, of alkaline protease has been

confirmed in the case where 168PHsigA or 168PFsigA was employed as a host cell, as compared with the control 168 strain (wild type).

[0072]

[Table 10]

Primer	Nucleotide sequence	SEQ ID NO.
S237pKAPpp -F	ACTTTAAAAATATTTAGGAGGTAATATGAAGAAACCGTTGGGGAAA	22
KAPter -R (BglII)	GGGAGATCTTCAGCGATCTATTTCTCTTTTTC	23
S237ppp -F2 (BamHI)	CCCGGATCCAACAGGCTTATATTTA	24
S237pKAPpp -R	TTTCCCCAACGGTTTCTTCATATTACCTCCTAAATATTTTTAAAGT	25
K38matu -F2 (ALAA)	GCTCTTGCAGCAGATGGATTGAACGGTACG	26
SP64K38 -R (XbaI)	TTGGTCTAGACCCCAAGCTTCAAAGTCGTA	27
S237ppp -R2 (ALAA)	TTCAATCCATCTGCTGCAAGAGCTGCCGG	28

[0073]

[Table 11]

Host	Amount of produced (secreted) alkaline protease (relative value)
168 (wild type strain)	100
168PHsigA	129
168PFsigA	130

[0074]

Example 5 Evaluation of *Bacillus subtilis* mutant strain in terms of alkaline amylase production (secretion) performance

168PHsigA and 168PFsigA, exhibiting enhanced alkaline cellulase and alkaline protease productivities confirmed in Examples 3 and 4, were evaluated in terms of production

performance of other proteins and polypeptides. Specifically, among others, alkaline amylase production performance of the above two strains was investigated by use of the genus *Bacillus* in the following procedure.

A genome DNA sample, serving as a template, extracted from *Bacillus* sp. KSM-K38 (FERM BP-6946) and a primer set of K38matu-F2 (ALAA) and SP64K38-R (*Xba*I) shown in Table 10 were employed, to thereby amplify through PCR a 1.5 kb DNA fragment (J) encoding alkaline amylase (Appl. Environ. Microbiol., 67, 1744, (2001)) having an amino acid sequence represented by SEQ ID NO: 19. Separately, a genome DNA sample, serving as a template, extracted from *Bacillus* sp. KSM-S237 (FERM BP-7875) and a primer set of S237ppp-F2 (*Bam*HI) and S237ppp-R2 (ALAA) shown in Table 10 were employed, to thereby amplify through PCR a 0.6 kb DNA fragment (K) containing a promoter region of an alkaline cellulase gene (JP-A-2000-210081) and a region encoding a secretory signal sequence. Subsequently, SOE-PCR was performed by use of a primer set of S237ppp-F2 (*Bam*HI) and SP64K38-R (*Xba*I) shown in Table 10 and the thus-obtained two fragments (J) and (K) in combination as templates, to thereby produce a 2.1 kb DNA fragment (L) in which an alkaline amylase gene was ligated downstream of the promoter region of an alkaline cellulase gene and the region encoding a secretory signal sequence. The thus-produced 2.2 kb DNA fragment (L) was inserted into the *Bam*HI-*Xba*I restriction enzyme cleavage site of a shuttle vector pHY300PLK (yakult), to thereby construct a plasmid

pHYK38 (S237ps) for evaluating alkaline amylase productivity.

The thus-constructed plasmid pHYK38 (S237ps) was introduced to each of the 168PHsigA and 168PFsigA, and to *Bacillus subtilis* 168 serving as a control through the protoplast transformation method. The cells were shake-cultured for five days, and other conditions were the same as employed in Example 3. After completion of culturing, cells were removed through centrifugation, and alkaline amylase activity of the supernatant obtained from the culture was determined, thereby calculating the amount of the amylase secreted from the cells during culturing; i.e., the amount of the extracellularly produced amylase. As is clear from Table 12, more effective production, or secretion, of alkaline amylase has been confirmed in the case where 168PHsigA or 168PFsigA was employed as a host cell, as compared with the control strain 168 (wild type). Thus, it was revealed that the aforementioned mutant strains were employed effectively in producing a variety of proteins and polypeptides.

[0075]

[Table 12]

Host	Amount of produced (secreted) alkaline amylase (relative value)
168 (wild type strain)	100
168PHsigA	189
168PFsigA	182